Synthesis and Purification of (W48F/Y72F/Y108W)AzM(II) and [Re(I)(CO)$_3$(4,7-dimethyl-1,10-phenanthroline)(H107)]AzM(II) (Az = azurin; M = Cu, Zn).

The mutations W48F, Y72F, H83Q, Q107H, Y108W were carried out stepwise using Invitrogene primers and a QuickChange® kit (Stratagene). The modified DNA plasmid (verified sequencing) was incorporated into BL21(DE3) *E. Coli* (Novagene). Bacteria were grown in a standard LB broth to an optical density of ~2. After harvesting, the cells were subjected to lysis by the osmotic shock method. The periplasmic extrudate was buffered with 25 mM NaOAc pH 4.5, 5 mM of either Zn(II) or Cu(II) acetate, and allowed to sit for 3 days at 4^o C. The precipitate was removed by centrifugation and the supernatant liquid concentrated by an Amicon Ultrafiltration (Millipore) device using an YM-10 membrane (Millipore). The concentrated periplasmic extrudate was then washed several times with 25 mM NaOAc pH 4.5 to remove remaining DNA. Azurin was purified and labeled as previously reported (Di Bilio *et al., JACS* **1998**, *120*, 7551; Di Bilio *et al.; JACS* **2001**, *123*, 3181). Azurin was analyzed by mass spectrometry (ESI/MS): found 13912.8; calcd (apo) 13913.8. The protein was also characterized by absorption spectroscopy (λ_{max} = 628 nm, Figure 1) and by SDS-Page gel electrophoresis (Pharmacia). ESI/MS on [Re(I)(CO)$_3$(4,7-dimethyl-1,10-phenanthroline)(H107)]- AzCu(II) (ReAz) yielded 14390.0, calcd (apo) 14391.0. The UV-Vis absorption spectrum of ReAz is shown in Figure 1. The Zn(II) derivative of ReAz was produced in an identical fashion.

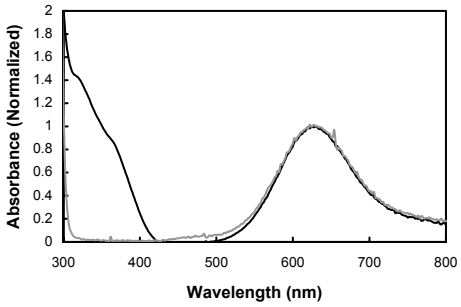

Figure 1. UV-vis absorption spectra of (Q107HW48F/Y72F/Y108W)AzCu(II) (—) and [Re(I)(CO)$_3$(4,7-dimethyl-1,10-phenanthroline)(H107)](W48F/Y72F/Y108W)AzCu(II) (╼).

Fluorescence spectrum of (Q107HW48F/Y72F/Y108W)AzCu(II)

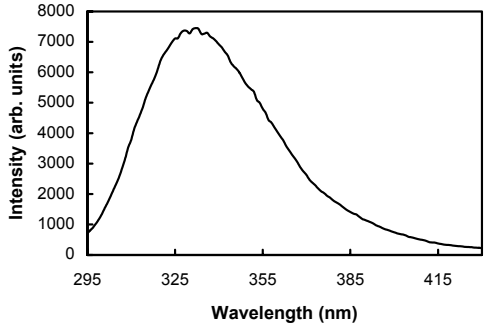

Figure 3. Fluorescence spectrum (measured with a Hitachi F-4500 fluorimeter) of (Q107HW48F/Y72F/Y108W)AzCu(II) in 50 mM phosphate buffer; λ_{ex} = 280 nm, λ_{max} = 330. The emission maximum of (Q107HW48F-/Y72F/Y108W)AzCu(II) is red shifted relative to native azurin (λ_{max} = 307; Finazzi-Agno *et al., Biochemistry* **1970**, *9*, 2009). The maximum of the (W108)Az emission spectrum is in the range for tryptophan residues in proteins in polar environments (Kroes *et al., Biophys. J.* **1998**, *75*, 2441; Clayton *et al., Eur. J. Bipohys.* **2002**, 319; Gilardil *et al., Biochemistry* **1994**, *33*, 1425).

EPR spectra of [Re(I)(W108$^{\bullet}$)AzZn(II)

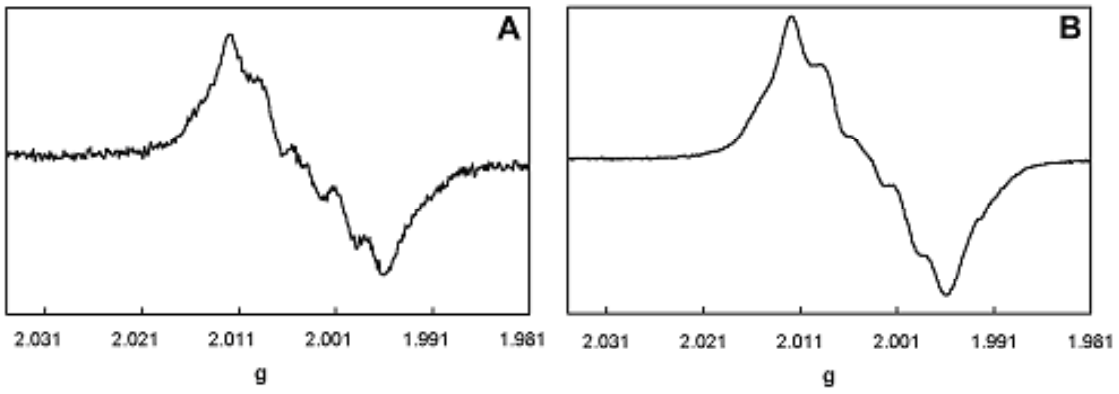

Figure 5. X-band EPR spectra of Re(I)Az(W108$^{\bullet}$)Zn(II). (**A**) Spectrum generated from a solution containing 300 μM of Re(I)Az(W108)Zn(II)/saturated $[Co(NH_3)_5Cl]Cl_2$ in 50 mM $NaHCO_3$, pH 9.8 (ν = 9.5369 GHz, modulation amplitude = 0.2 mT, microwave power ~ 200 μW) (**B**) Spectrum generated from a solution containing 300 μM of Re(I)Az(W108)Zn(II)/saturated $[Co(NH_3)_5Cl]Cl_2$ in 50 mM KP_i pH 7.16 (ν = 9.4886 GHz, modulation amplitude = 0.2 mT, microwave power ~ 200 μW). The EPR spectrum remains largely unchanged between pH 7-10.

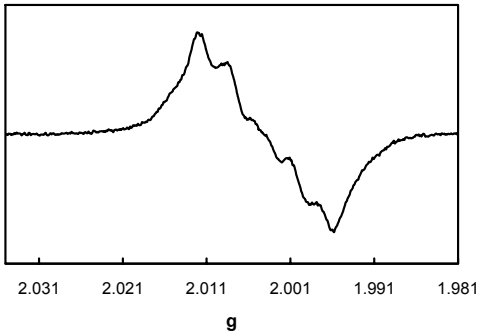

Figure 4: EPR spectrum of Re(I)Az(W108$^{\bullet}$)Zn(II) at 77 K in 50 mM KP_i/D_2O (pD = 7.5). Deuterium enrichment was achieved by diluting/concentrating ReAz with D_2O/KP_i buffer).

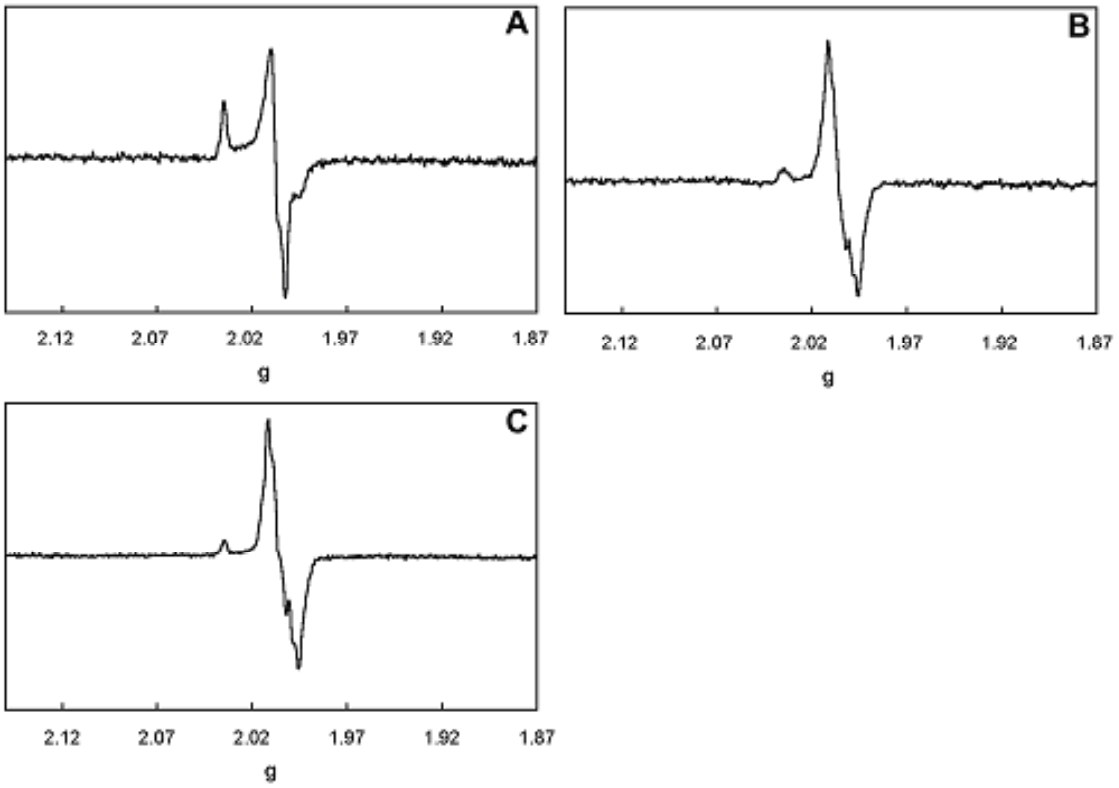

Figure 6: EPR spectra of ReAz(W108$^{\bullet}$)Zn(II) at 77 K generated from 300 μM Re(I)Az(W108)Zn(II)/saturated $[Co(NH_3)_5Cl]Cl_2$ under air in various media. At all pH values, photolysis in air lead to formation of a peroxyl Trp-based radical shown in Figure 6 (Sahlin *et al., J. Biol. Inor. Chem.* **2002**, *7*, 74). (**A**) 50 mM NaOAc pH 4.03 (ν = 9.4799 GHz, modulation amplitude = 0.2 mT, microwave power ~ 200 μW); (**B**) 50 mM KP_i pH=7.16 (ν = 9.4566 GHz, modulation amplitude = 0.2 mT, microwave power ~ 200 μW); (**C**) 50 mM $NaHCO_3$ pH 9.80 (ν = 9.4645 GHz, modulation amplitude = 0.2 mT, microwave power ~ 200 μW). Interestingly, the radical in pH 4.03 acetate buffer appears to be much more reactive towards oxygen than the radical at higher pH.

Density Functional Calculations of Trp radicals *g* tensor.

The main values of the *g* tensors of three tryptophan radicals were calculated using the method of Schreckenbach and Ziegler (Schreckenbach *et al,. J. Phys. Chem. A* **1997**, *101*, 3388) via the ADF package (te Velde *et al., J. Comput. Chem.* **2001**, 22, 931-967; Fonseca Guerra, C. *et al*., *Theor*. *Chem*. *Acc*. 1998, *99*, 391-403; ADF2003.01, SCM, *Theoretical Chemistry*, Vrije Universiteit, Amsterdam, The Netherlands, http://www.scm.com) using the Becke gradient correction (Becke, A. D. *Phys. Rev. A.*, **1988**, *38*, 3098) and Perdew correlation term. The values were scaled according to a survey of a variety of radicals which indicated that the theoretical values should be linearly scaled to achieve much better agreement with experimental values (Un, in preparation). Such scaling produced calculated g-values that were well within ± 0.0004 of the experimental values. The three models were: (A) N-protonated indole radical, (B) a formic acid-deprotonated indole radical hydrogen-bonded complex and (C) a deprotonated indole radical (see Table 1). All three structures were geometry optimized with the Gaussian94 package (Gaussian 94, Revision D.2, M. J. Frisch, G. W. Trucks, H. B. Schlegel, P. M. W. Gill, B. G. Johnson, M. A. Robb, J. R. Cheeseman, T. Keith, G. A. Petersson, J. A. Montgomery, K. Raghavachari, M. A. Al-Laham, V. G. Zakrzewski, J. V. Ortiz, J. B. Foresman, J. Cioslowski, B. B. Stefanov, A. Nanayakkara, M. Challacombe, C. Y. Peng, P. Y. Ayala, W. Chen, M. W. Wong, J. L. Andres, E. S. Replogle, R. Gomperts, R. L. Martin, D. J. Fox, J. S. Binkley, D. J. Defrees, J. Baker, J. P. Stewart, M. Head- Gordon, C. Gonzalez, J. A. Pople, *Gaussian*, *Inc*., Pittsburgh PA, 1995.) using the Becke three parameter functional (Becke, A. D. *Phys. Rev. A.*, **1988**, *38*, 3098 Becke, A. D. *J. Chem. Phys.*, **1993**, *98*, 5648) and the Lee, Yang and Parr (Lee *et al. Phys. Rev. B.*, **1988**, *37*, 785-789) correlation functional with a minimum basis set of 6-31*. As a point of comparison, *g* tensors for two RNR based tryptophan radicals were included: W111, with two nearby glutamate residues and W177, a tryptophan with a single nearby aspartamate (Bleifuss *et al. Biochemistry* **2001**, *40,* 15362).

Table 1: Calculated *g* tensor for models and protein tryptophan radicals. W108 (Az) is from the present work.

Radical	g_z	g_y	g_x
A	2.00247	2.00271	2.00345
B	2.00247	2.00296	2.00359
C	2.00247	2.00299	2.00380
W108 (Az)	2.00221	2.00271	2.00355
W111 (RNR)	2.00210	2.00240	2.00330
W177 (RNR)	2.00230	2.00250	2.00350

The trend towards lower g_x values with hydrogen bonding is common to radical possessing lone-pair electrons (see, for example Ivancich, A. *et al. JACS* **2002**, *121*, 5743; Un, S., *et al. JACS* **1995**, *117*, 10713). The RNR based tryptophan radicals (W111, W177 in Table 1) support this picture and imply that W108$^{\bullet}$ is likely interacting with a nearby H-donor, but more weakly so than for either RNR radical.

Time resolved multi-wavelength spectrophotometer.

The excitation source was a Spectra-Physics Nd:YAG. The Nd:YAG unit produces pulses at 1064 nm with a 10 ns duration. These pulses are tripled, to put out pulses at 355 nm of approximately 300 mJ/pulse. The laser beam intensity was reduced by 90% with a partial reflectance mirror and the power further modulated by a polarizer to give pulses at the sample of between 1 and 3 mJ/pulse. The samples were excited with this pulse, and probed with a microsecond flash lamp. The flash lamp exhibits a 4 μs jitter, which means that the temporal resolution of the instrument had an error of 4 μs. Based on the distance the light must travel and inherent delays in the electronics, the minimum time between a pulse from the laser and a probe from the flash lamp was 16 μs. The delay between probe and pump was set by a Standford Research Systems digital delay generator (model DG535). The probe light was collected and transferred by a fiber optic line to a Spex 270M monochromator. A grating with a 500 nm blaze and 300 grooves/mm in the monochromator gave a dispersal of ~ 300 nm. The light from the probe was split before the sample to provide a reference light source, allowing for a subtraction of the sample and a blank to give ΔOD transient absorption spectra. Dispersed light from the probe and reference was sent to a cooled (-30 ^{o}C) Princeton Instruments 2-strip photodiode array (DPDA-1024) and the resulting information handled by a Princeton Instruments ST-116 controller. The data was processed by Lab View software virtual instruments. In order to calibrate the spectra, band pass filters were placed in front of the monochromator while the probe light was on. These filters allow only a narrow range of light to pass through, so by assigning the maximum of each filter to a pixel on the DPDA, the calibration curve for the monochromator can be derived. Before each collection cycle, transient absorption spectra of $MV^{+\bullet}$ (generated in a solution of $[Ru(bpy)_3]Cl_2$ with MV^{2+} in ethanol) was obtained to verify that the instrument is working correctly.

Optical spectra for [Re(CO)$_3$(4,7-dimethyl-1,10-phenanthroline)(H107)]$^{\pm}$AzZn(II).

The time resolved optical spectra acquired for Re(W108$^{\bullet}$)Az over a large pH range and in deuterated buffer are shown in Figure 8).

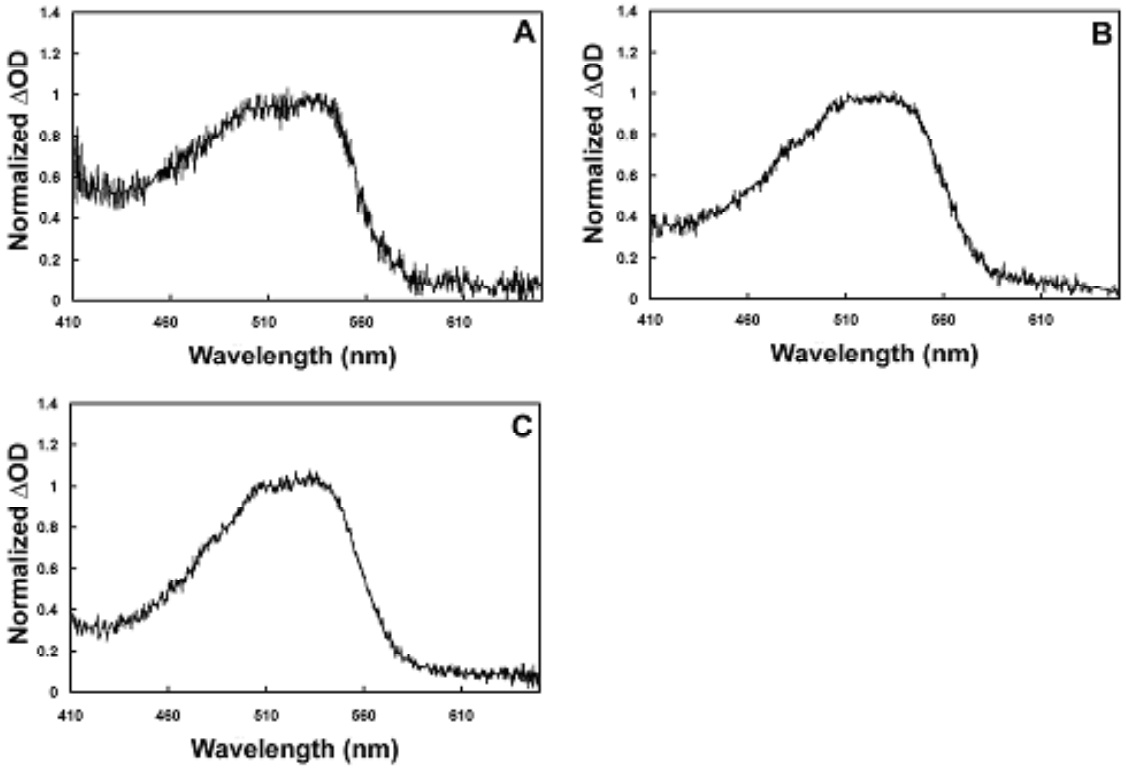

Figure 8: Absorption of ReAz(W108$^{\bullet}$)Zn(II), 20 μs after excitation in (A) 30 μM ReAz(W108)Zn(II) in 50 mM NaOAc pH 4.03; (B) 42 μM ReAz(W108)Zn(II) in 50 mM KP$_i$ pD 7.5; (C) 44 μM ReAz(W108)Zn(II) in 50 mM $NaHCO_3$ pH 9.80.

Reduction of Re(W108$^{\bullet}$)AzCu(II) by Mo(IV).

To establish the reduction potential for the radical, we reduced the radical with $K_4Mo(CN)_8$. We used the AzCu(II) in order to establish (by spin-integration) the concentration of radical formed. Since the radical is generated by photolysis concomitant with freezing, distribution of the radical in solution is not uniform. Therefore, the values obtained from spin integration are approximate. The result of this experiment is shown in Figure 7. The broad signal at g ~ 2.057 is due to the Cu(II) center. The features between g ~ 2.002-1.956 are due to a Mo(V) species (McGarvey, B. R. *Inorg. Chem.* **1965**, *5*, 476).

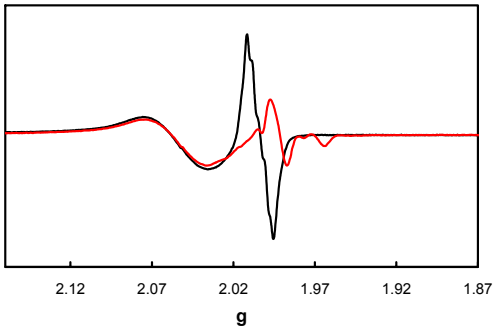

Figure 7: (—) EPR spectrum of ~ 40 μM ReAz(W108$^{\bullet}$)Cu(II) at 77K in 50 mM KP_i pH 7.2 (—) EPR spectrum of the same sample after 8 min at RT and with 1.5 mM $K_4Mo(CN)_8$ (ν = 9.3714 GHz, modulation amplitude = 0.2 mT, microwave power ~ 200 μW).